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Anti zona pellucida antibodies in follicular fluid and outcome of ICSI

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ABSTRACT

Objective: To assess the correlation of the presence of AZA in the follicular fluids (FFs) of women who underwent ICSI, and etiology of infertility as well as multiple puncture of ovaries, and also the relation of presence of AZA with the results of ICSI, as the main outcome, to realize if ICSI can bypass the possible effect of AZA on reproduction.
Materials and Methods: In this prospective one year study, follicular fluids were evaluated from 96 infertile women, who referred to Avesina clinic for ICSI, including (19-40 years old, 31.5±5.1), that based on the etiology of infertility, 80 women were recognized with explained infertility whereas 16 were recognized with unexplained infertility. All FFs were evaluated by ELISA test.
Results: Twenty of samples (20.8%) were AZA positive. In patients with unexplained infertility, AZA antibody in follicular fluid, was significantly higher than the group with proven etiology of infertility (P value =0.001). Additionally in 20.4 % of patients, who had been punctured previously, AZA was detected in their FFs which are statistically similar to the patients who were punctured for the first time. In this regard, there were no significant correlation between the presence of AZA and the number of oocytes, embryos, fertilization rate and clinical pregnancy rates in patients who underwent ICSI.
Conclusions: The high incidence of AZA in infertile women, especially the ones with unexplained infertility has to be considered .The relation between the presence of AZA and repeated puncture of ovaries is still debatable. The clinical significance of AZA in follicular fluid in the outcome of in- vitro fertilization was suggested in some studies. Correlation between fertilization rate of AZA and the number of oocytes and positive AZA still remains controversial. However, it seems that microinjection could bypass the possible effects of AZA on fertilization, the number of embryos and clinical pregnancies. Determinations of AZA are highly recommended in evaluation of infertile couples especially in patients with unexplained infertility for making appropriate decision regarding the need for ICSI.
Key Words: Anti-zona antibody (AZA), unexplained infertility, Follicular fluid, Repeated IVF attempt, ICSI.

INTRODUCTION

Auto antibodies to zona pellucida (AZA) seem to be important autoantibodies implicated in the etiology of infertility (1), especially unexplained infertility (2, 3). The acellular zona pellucida (ZP) surrounds the egg while ovulation occurs and remains in place until the implantation, contains receptors (ZP1, ZP2, ZP3) (4) for sperms which are- with some exceptions- species-specific (5). The initial contact between the sperm and the oocyte is a receptor-mediated process (6). Specific antibodies against these acidic glycoproteins are able to inhibit sperm attachment and penetration into oocyte and may be the cause of natural or artificial fertilization (7). Antigens of Zona pellucida seems to be appropriate for anti -fertility vaccines, because AZA may block both

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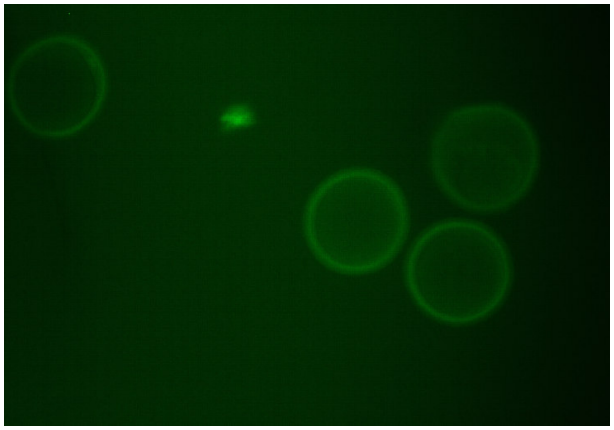

Figure 1. Immuno- fluorescent staining directed against intact zona pellucida

fertilization and implantation and low amounts of antibody would be sufficient for contraception because of the small number of mature eggs with zona present at any time (8).Various studies have impressed the incidence of AZA among 7.5-36.5 % infertile patients (1, 9). There are some argument about the possible role of antibody on fertilization failure in IVF programs(10), however, some studies show higher incidence(39-91%) of antibody in the patients with IVF failure (9,12) . The increased levels of serum and AZA are very often (detected) after repeated unsuccessful IVF (13, 14). However, AZA may coexist with other fertility disorders (15). The main purpose of this study is to evaluate the prevalence of this antibody in FFs of infertile patients, finding the probable relation to the etiology of infertility, correlation between the number of previous punctures and the presence of antibody, and finally the effect of antibody on the outcome of ICSI to know if it is important to search these antibodies as a valuable diagnostic tool in infertile patients for making appropriate decision for the need for ICSI to treat even sever case of immunological infertility.

Table 1. AZA regarding to the etiology of infertility in patients undergoing ICSI

	Total (n = 96)	Positive AZA* (n = 20)
Male factor (%)	56 (58.3)	11 (19)
Tubal factor (%)	10 (10.4)	2 (20)
Ovulatory factor (%)	7(7.2)	–
Tubal and male factor (%)	3 (3.1)	–
Ovulatory and male factor (%)	4 (4.1)	–
Unexplained factors (%)	16 (16.6)	7 (43)

* Anti Zona Antibody

METHODS AND MATERIALS

Ethics of Experimentation:

Investigations have been approved by ethical committee of Avesina Research institute and the relevant consent has been obtained from patients.

Investigated patients:

This cross sectional one year study was conducted in a prospective manner in Avesina Research Institute. The entire women (n = 96), mean age (±SD) was 31.5±5.1, (min=19; max=39). who met the clinic, consecutively were considered in our study, assessed by history, physical exam, routine lab measurement (plasma FSH, LH and E2 at 3rd day of cycle, TFT, Prolactin, midluteal progesterone level), sperm analysis and imaging (sonography, HSG) to categorize infertile patients as explained (n=16) and unexplained (n=80).

Women met the inclusion criteria, if 1) they were younger than 40; 2) They were the candidates for fresh ICSI according to the indications, 3)They didn't show the history of rheumatology and immunology diseases.

Measurements:

All FFs were assessed for the presence of AZA by ELISA test to get the prevalence of AZA in infertile patients. Moreover, the prevalence of AZA was compared between patients with unexplained and explained infertility, and also it has been compared based on the number of previous punctures. We also compare the outcome of ICSI between two groups of AZA positive and AZA negative.

Table 2. Features of treatment cycles among two groups according to AZA results

	AZA Negative (n =76)	AZA Positive (n=20)	P-value
Age (years)	31.4±5.06	32.55±5.1	NS
HMG ampoules (n)	29.5±21	31.5±22	NS
Day of HCG stimulation (days)	11.3±2.1	11.3±1.5	NS
No of Oocytes (n)	8.4±5.65	6.4±4.03	NS (0.073)
No of embryos (n)	4.78±4.06	3.3±2.8	NS (0.073)
Fertilization (%)	53±30	57±32	NS
Clinical Pregnancy (%)	32(42.1)	8(40)	NS

Protocol of stimulation:

GnRH- a (Buserelin, Hoechst, Germany), (0.1 mg/24 hours) was administered from day 21 of the cycle. From day 3 of the next cycle, all patients were treated with human menopausal gonadotropin (hMG; Merional, IBSA, Switzerland). The initial dose of the administered gonadotropin was set at 150 IU / day and eventually increased by steps of 75 IU every 3-4 days while controlling follicular growth under ultrasound. Ovulation was induced by the intramuscular administration of 10000 IU of the human chorionic gonadotropin (hCG; Pregnyl ,Daroupakhshs,Iran) when the three leading follicles had reached a diameter of 18 mm. Oocyte retrieval was performed by transvaginal aspiration 36 hours after hCG administration and FFs were obtained following oocyte collection , and kept frozen until antibody evaluation.

ELISA test:

1-Production of Mouse Anti-human Zona Pellucida Antibodies:

Murine anti human zona antibodies were prepared as described earlier with minor modifications (16, 17). Balb/c mice were subjected to 4 intra-peritoneal injections with 4-6 cumulus-free live unfertilized oocytes from IVF or microinjection procedures, after obtaining consent from the respective couples. After fourth injection, 1/100 dilutions of the mouse sera were tested for AZA by immunofluorescent staining of intact zona on dissected live unfertilized oocyte using FITC conjugated goat anti mouse immunoglobulins (Sigma). Figure -1 shows immuno fluorescent staining directed against intact zona pellucida . Immunized mice were selected, sacrificed and their blood samples were collected and sera were separated by centrifugation.

2-Detection of human AZA in FF by a cell-based ELISA:

To detect the presence of AZA in the FF of our subjects an oocyte/embryo based on the ELISA was developed. In every test 5 unfertilized human oocytes (see above) were used. We used live cells against isolated zona pellucida ,due to better presentation of zona pellucida by live cells. The cells were initially washed once with sterile phosphate buffered saline (PBS) under embryologic loop and by mouth pipetting. Separate drops of PBS, each containing 5 oocytes/embryos were laid in Petri dishes. The first drop that served as negative control received only PBS and cells. To the cells in the positive control drop 1:100 dilution of the mouse anti-zona immune serum was added. Cells in test drops received individual undiluted patient FFs. After 30 minute incubation in the cold, cells in all drops were washed for three times with cold PBS by mouth washing under loop microscope. The positive control drop was then incubated for 30 minutes with peroxidase conjugated Goat anti-mouse Ig (Sigma) on ice. All other drops received the same treatment but with peroxides conjugated Goat anti-human Ig (Sigma). All cells were then washed for three times with PBS and were then transferred in 50 µl of TMB substrate solution into ELISA strip wells (Nunc) and incubated for 15 minutes in the dark followed by addition of 50 µl of stop buffer (20% sulfuric acid). The optical density (OD) of the reactions was measured in an ELISA reader at 492 nm wavelength.

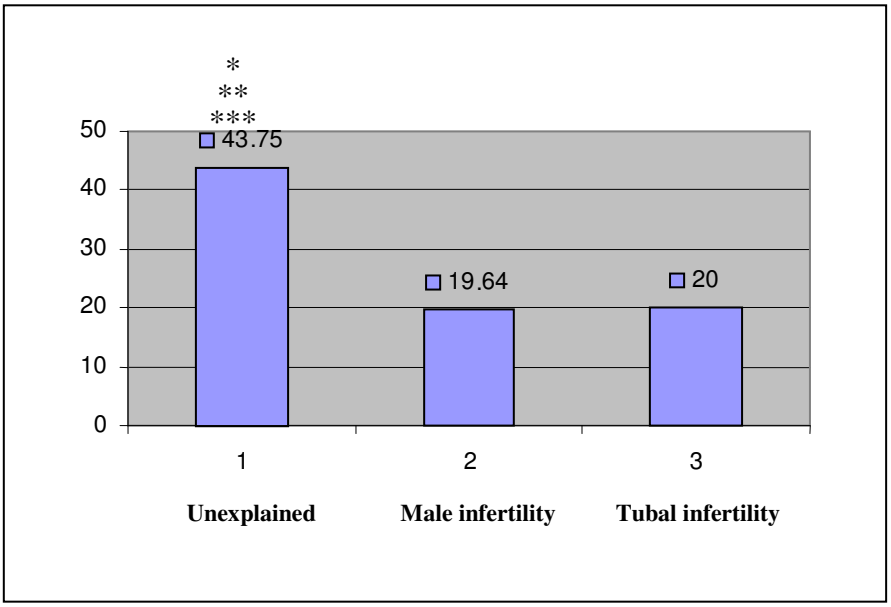

Figure 2. Comparison of frequencies (%) of AZA between unexplained, tubal and male factor infertility. * p= 0.001 vs Explained infertility, ** p= 0.045 vs male factor infertility, *** p= 0.004 vs tubal factor infertility

3-Validation of the ELISA results:

All the FF samples were also tested for presence of AZA using a commercial Anti Zona Antibody ELISA kit (Bioserve Diagnostics, Rostock, Germany) and all the results were confirmed.

Statistics:

Data were presented as mean ±SD for numerical variables and in percentage for categorized variables. The data analysis was performed with SPSS software. Statistical assessment of our results was performed using 1-sided and 2-sided Chi-Square test. A P- value of <0.05 was considered statistically significant.

RESULTS

According to the etiology of infertility, 80 (83.3%) of women had explained infertility including male factor (56%), tubal factor (10%), ovulatory factor (7%), tubal and male factor (3%), ovulatory and male factor (4%), whereas 16 (16.6%) had unexplained infertility. 20.8 % of samples (n=20) were positive for AZA according to the result of the IEA test. Table 1 shows the frequency of AZA regarding the etiology of infertility in patients undergoing ICSI.

We compare the frequencies of positive AZA between explained and unexplained groups in Figure-2. In patients with unexplained infertility, the prevalence of AZA antibody in follicular fluid was significantly higher than the group with proven etiology of infertility (43.75% vs 16.25%). Also, Figure- 2 shows comparison of frequencies of positive AZA between unexplained and tubal factor groups. In the group with unexplained infertility, AZA antibody in follicular fluid was significantly more frequent than the group with tubal factor infertility (43.75% vs 20%). Comparison of the frequencies of AZA between unexplained and male factor groups is shown in Figure -2. In the group with unexplained infertility, AZA antibody in follicular fluid was significantly shown more than the group with male factor Infertility (43.75% vs 19.64%).

Figure -3 shows the results according to the number of punctures. The results didn't show obvious relationship between number of previous punctures and frequency of AZA in FF.

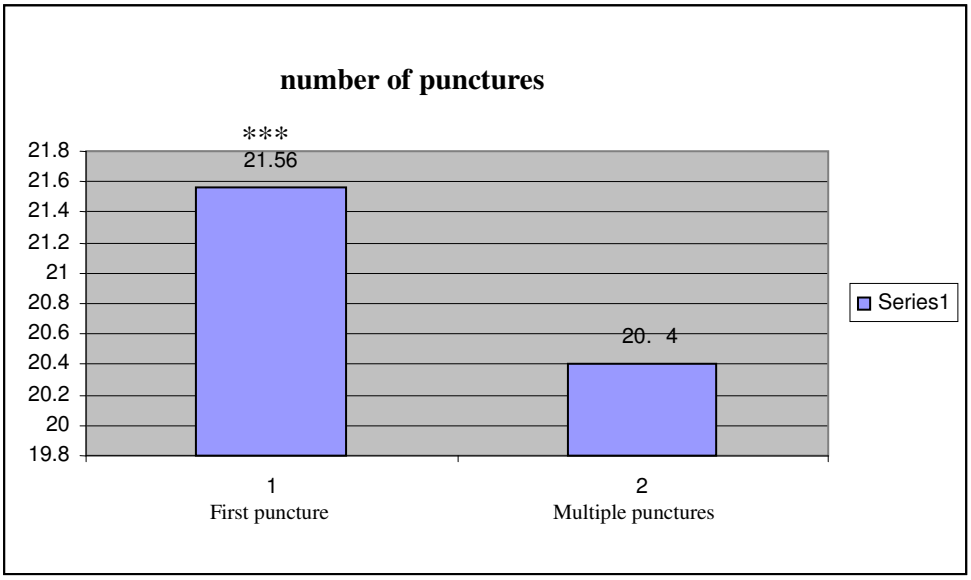

Figure 3. Comparison of frequency of AZA (%) according to the number of ovarian punctures. *** p vs multiple puncture= not significant

We evaluated the relation of age and frequency of AZA (Table 2). No statistically significant relation between age (mean ± SD) and frequency of AZA (%) were apparent (31.4± 5.06 years for AZA negative group and 32.55 ± 5.1 for AZA positive group).

Table 2 shows features of treatment cycles among two groups according to AZA results. As you can see in the table, there are no significant differences between two groups including AZA positive and AZA negative, regarding age, number of hMG ampoules, duration of follicular phase, number of oocytes, number of embryos, fertilization and the rate of clinical pregnancy.

DISCUSSION

Some studies suggest possible immuno-pathological mechanism for reproduction failure in patients with organ-specific auto antibodies. These auto antibodies like anti thyroid and anti ovarian antibodies are introduced as possible markers for reproduction failure (18-19). Absorption and degradation of the ova into the peritoneal cavity or in the reproductive tract and the subsequent exposure of the degradation products to the immune system, is the possible mechanisms for production of AZA, similar to that described by Mhaskar who demonstrated the presence of antibodies, specific to zona antigens, in tubectomized women. (20). There are few reports indicating the incidence of AZA in FF patients with infertility. However, presence of AZA in human sera and their characterization have been studied extensively (21, 22, 23). In this study we assessed the rate of AZA in FF of infertile women (20.8 %) in comparison with the previous findings in human sera. Some studies showed an anti – gamete antibodies (AGA) in high percentage of patients with unexplained infertility versus patients with proven etiology of infertility (1, 2, and 16). Positive fluorescence was found by Nishimoto for AZA, in 7.4% from patients with unexplained infertility, in 1.6% from patients with proven etiology of infertility, but in none of age-matched control subjects (fertile women and men) (2). AGA (anti-gamete antibodies) were found by Moustafa in 45% patients with unexplained infertility as a possible cause of infertility (14). AZA were found in 3 of 10 patients with low response to ovarian stimulation in Hovav study (15). The presented study proved the results of previous studies and showed high incidence of AZA in women with unexplained infertility. These results suggest an

etiologic association of antibody with infertility. Nishimoto found that, AZA may be generated during the aging process (2), but we didn't find any relation between age and frequency of AZA in our study.

Correlation between fertilization rate and positive AZA still remains controversial. However, there is no evidence regarding correlation of the presence of AZA and the number of oocytes, embryos and pregnancy rate. Some studies have shown a significant prevalence of AZA in patients with fertilization failure and the ones with low fertilization rate. Mustafa investigated the effect of anti-gamete antibody on the success of IVF-ET and suggested that fertilization rates are lower in immunological and unexplained infertility patients than in tubal infertility patients, being statistically significant. Moreover, pregnancy rates are lower in immunological and unexplained infertility patients than in tubal infertility patients after IVF-ET (14). Also, Papale assessed the relation of AZA in follicular fluids and fertilization rate in IVF. Out of 55 follicular fluids analyzed, 36.3% were positive to the test and no fertilization was observed in oocytes from these follicles, while 63.6% were negative, and the oocyte fertilization rate associated with these was 51.4%. It was concluded that, the presence of AZA was positively correlated with the degree of fertilization failure (9). A significant prevalence of AZA detected by ELISA by Ivanova in follicular fluid in the patients with fertilization failure (39.13%) and with low fertilization rate (12). However, no correlation between the presence of AZA in serum, and failure of fertilization in IVF was found in Curtis study, and detection of AZA has no role in the prediction of IVF (10). Furthermore, higher proportion of AZA activity in serum and follicular fluid was observed in pregnant patients by IVF, than that observed in non-pregnant in Mantzavinos study and there was found no statistically significant differences for the mean number of oocytes retrieved and fertilized irrespective of the presence of antibodies in serum or in follicular fluid. However, higher fertilization rates were observed in serum or follicular fluid antibody negative patients (68.2% and 71.2%, respectively) than those who were tested positive (36.6% and 65.4%, respectively) (11).

The influence of anti-sperm (ASA), anti-phospholipids (APA), and antizonal (AZA) antibodies on in vitro fertilization (IVF) results and the need for intracytoplasmic sperm injection (ICSI) were assessed by Mardesic. He concluded that immunologic infertility can be treated by IVF with very good results, but in the most important group, the women with AZA, ICSI without any delay is recommended (18).

In this study, all of the treatment cycles were ICSI, there was no significant relation between the presence of AZA and fertilization rate. There were also no significant correlations between the presence of AZA and the number of oocytes, embryos, fertilization rate and clinical pregnancy rates.

Some studies propose trigger of an autoimmune process due to micro trauma induced by repeated punctures of ovarian follicles, which can result in the production of auto-antibodies in women subjected to in vitro fertilization (IVF).

Gobert, showed that anti-ovary antibodies -after attempts at human in vitro fertilization- induced by follicular puncture rather than hormonal stimulation. High concentrations of these antibodies have been found in women who have had several IVF attempts and they appear to correlate with reduced chances of pregnancy. (24). Ulcova-Gallova, assessed the levels of zona pellucida antibodies in 250 women divided into four groups according to the number of recurrent IVF failures (1-4) were analyzed and compared with results of a control group of 211 unexplained infertile women never treated by IVF. These tests showed increased occurrence of zona pellucida antibodies in women after repeated IVF. AZA were found in 20% after one unsuccessful IVF, but in 64% after two, in 91% after three and in 4 of 5 cases after four IVF failures. The results show evolution of autoimmune process due to repeated ovarian intervention during oocyte collections (25). Barbarino-Monnier mentioned that micro trauma induced by repeated puncture of ovarian follicles can result in the production of auto -antibodies in women submitted to IVF (26). Furthermore, in the other study, Monnier-Barbarino assessed anti-ovarian antibodies (AOA) in serum samples at various times of in vitro fertilization (IVF) attempts to determine whether ovarian stimulation

could result in the production of such auto-antibodies in women. The study showed the absence of influence of endogenous or exogenous ovarian stimulation by gonadotropins on anti-ovarian autoimmunity (27). In contrast, Hovav assessed the presence of antizona pellucida autoantibodies in patients undergoing IVF in relation to low ovarian response, multiple IVF attempts and unexplained infertility suggested an association between antizona pellucida antibodies and suboptimal response to gonadotrophins. None of the patients with multiple IVF attempt demonstrated measurable level of anti zona antibody in the study group. It was indicated that repeated stimulation and puncture of ovaries in IVF procedures do not elicit autoimmunity to gametes (15). The presented study showed no correlation between previously repeated punctures and measurable level of AZA in follicular fluid and it presented no respective role of repeated puncture of ovaries to induce such an autoimmune response to produce AZA higher than patients who were punctured for the first time.

CONCLUSION

High incidence of AZA and long-term resistance to treatment in women with unexplained infertility, suggests an etiologic association of the antibody with infertility which may closely correlated with inhibition of sperm-egg interaction by AZA, produced in these women. In conclusion, the presence of these antibodies, could have adverse effects on the outcome of assisted reproductive techniques (ART). So, determinations of AZA are highly recommended in the evaluation of infertile couples especially in patients with unexplained infertility. Moreover, ICSI is a reliable option in these patients which prevents the possible effects of AZA on the number of oocytes, embryos and fertilization rate.

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